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November 3, 2003

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VIA FACSIMILE
(confirmation by COURIER)

RE : UNIVERSITE DE MONTREAL et al.
International patent application
No. PCT/CA03/00499 filed on April 4th, 2003
Our ref.: 000711-0025

Dear Sirs:

A- We hereby refer to the Demand filed under Article 31 PCT sent simultaneously with this fax and respectfully request that, as International Preliminary Examiner Authority, you proceed to a substantial examination of the above application.

B- We would also like you to take into account the following voluntary amendment made under the provisions of Article 34 PCT.

IN THE DESCRIPTION:

Cancel pages 3, 6, 9, 10, 13, 16, 17, 18 and 21 of the description presently on file, and insert the new corresponding pages 3, 6, 9, 10,13, 16, 17, 18 and 21 enclosed herewith.

IN THE CLAIMS:

Cancel page 39 containing original claims 10 to 17, and pages 41 to 43 containing original claims 26 to 38 presently on file, and insert the new corresponding pages enclosed herewith.

C- As will be noticed:

- the word "catalyst" has been replaced by --reagent-- on page 3, line 20, and page 9, line 26 of the description (the same correction has been made to claim 29);
- the term "con^3" has been replaced by --cm^3-- on page 6, lines 11 of the description;
- the expression "diacid dichloride" has been replaced by --diacid chloride -- on page 9, lines 17, 19 and 23 of the description (the same correction has been made to claim 34);
- the word "dichlorine" has been replaced by --dichloride-- on page 10, lines 17 and page 13, line 18 and 21 of the description (the same correction has been made to claims 12 and 27);
- the word "dibromine" has been replaced by --dibromide-- on page 10, line 17 and on page 13, line 18 of the description (the same correction has been made to claims 12 and 27);
- the word "polymerization" has been replaced by --polycondensation-- on page 16, line 16, on page 17, line 2 and on page 21, line 28 of the description; and
- the word "copolymerization" has been replaced by--polycondensation-- on page 18, line 13 of the description.

The above-mentioned modifications were made to the description essentially to correct obvious typographical and/or clerical errors.

As the Examiner will also note, the chemical name "1-(3-dimethylaminopropyl)-3-ethylcarbodiimide" has been inserted after the reference to its abbreviation EDC on page 10, line 1 of the description and in claim 35. This modification was done to remove any ambiguity as to the meaning the abbreviation EDC which has been inadvertently identified as meaning "ethylene dichloride" in claim 35. Such a correction is obvious for any one skilled in the art, especially upon reading page 10, line 1 which indicates that EDC is a chemical "equivalent" to DCC. It is obvious that such an equivalent is not and could not be ethylene dichloride.

As the Examiner will further note, the chemical formulae (V), (VI) and (VII) have been corrected on page 10 and 13 of the description. More specifically, the "-OH" group has been replaced by -- =O -- on the right hand terminal end of the polymeric backbone of chemical formulae (V) and (VI) on page 10 of the description. The chemical formula (VII) has also been corrected in such a way that the "$=CH_2$" has been replaced by a -- =O – group on page 13 of the description.

Once again, the above-mentioned modifications were made essentially to correct errors that were noticed in the formulae, which errors are obvious in view of the names of the compounds as given adjacent to the formulae.

Respectfully submitted,

ROBIC

Thierry Orlhac

DD/TO/lr

Encl. - pages 3, 6, 9, 10,13, 16, 17, 18 and 21;

- pages 39, 41 to 43 containing claims 10 to 17 and 26 to 38.

c) reacting the polyethylene glycol having terminal dichloride acid functions obtained in step b) with the PLA-PEG-PLA triblock polymer obtained in claim 34 by making use of polycondensation reaction so as to obtain a multiblock copolymer according to the invention.

A fifth object of the invention is to provide an improved method for preparing a PLA-PEG-PLA multiblock copolymer of formula (I):

$$ABA\text{-}(c\text{-}ABA)_n\text{-}c\text{-}ABA \qquad (I)$$

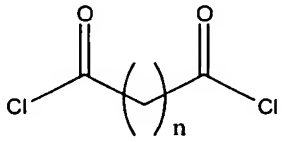
wherein

- n is a number equal or higher than 2;
- ABA is a PLA-PEG-PLA triblock; and
- c is a carboxylic diacid.

said method comprising the steps of:

a) preparing a PLA-PEG-PLA triblock;

b) mixing the PLA-PEG-PLA triblock prepared in step a) with a diacid of formula (II):



(II)

wherein n is a number equal to or greater than 1; and

c) subjecting the mixture of step b) to a polycondensation reaction with the presence of a dicyclohexylcarboxydiimide reagent and/or a chemical equivalent thereof, said catalyst improving the efficiency of the reaction, thereby allowing to obtain the requested multiblock copolymer.

A sixth object of the invention is to provide a method for delivering a pharmaceutical compound into a mammal, said method comprising the step of:

administering to the mammal a stealthy polymeric biodegradable nanosphere according to the invention loaded with a therapeutically effective amount of the pharmaceutical compound.

Figure 11 is the chemical formula of a multiblock polymer according to the invention.

Figure 12 A is a micrograph representing the nanosphere according to the invention after the release period of twenty-nine days.

Figure 12 B is a micrograph representing a nanosphere according to the invention that underwent degradation in a phosphate buffer at 37°C.

Figure 13 is a graph representing the weight loss of the bulk polymer used according to the invention.

Figure 14 is a graph representing the typical pore size distribution of nanospheres according to the invention.

Figure 15 is a bar graph representing the porosity (cm^3/g) of nanospheres made of various blends of PLA and multiblock polymers.

Figure 16 is a graph representing the proliferation of B16 cells in the presence of different components.

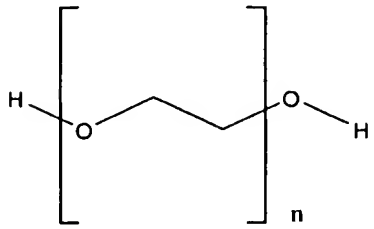
Figure 17 is graph representing the *in vitro* release of Rhodamine from nanospheres according to the invention in a phosphate buffer at 37°C.

Figure 18 is a graph representing the plasmatic concentration of Rhodamine after IV injection of nanosphere according to the present invention.

Figure 19 is a graph representing the concentration of Rhodamine in different organs.

Figure 20 are bar graphs representing the behavior of phagocytic cells in the presence of stealthy nanospheres according to the present invention.

Monomer B (PEG) can be obtained from the following compound:

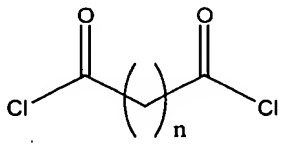


B

Wherein n represent a number between 200 and 2000.

Typically, the first step consists of mixing together one or several compounds of the A type with a compound of the B type. The compounds are polymerized by polycondensation under an inert atmosphere at a temperature of 160°C to 180°C for 2 to 6 hours. A tin-based catalyst such as tin octanoate or tetraphenyltin. The polymer ABA so obtained is dissolved in acetone and precipitated with water. The precipitate is then washed and dried.

The most common method for synthesizing a PLA-PEG-PLA multiblock copolymer from the ABA polymer is the method developed by Dupont in the seventies. Briefly, the ABA triblock polymer is placed in a round bottom flask in presence of a diacid chloride. Following a polymerization by polycondensation, and elimination of HCl, a multiblock ABA copolymer (ABA-c-ABA-c-ABA-c-ABA) is obtained. Suitable diacid chloride have the following formula (II):



(II)

Preferred diacid chlorides include: propanedioic acid, butanedioic acid, pentanedioic acid, etc.

Interestingly, the present inventors have found that the efficiency of the method is greatly improved when dicyclohexylcarboxydiimide (DCC) is used as a reagent in the reaction. Therefore, the present invention encompasses the use of DCC as well as chemical equivalents such as EDC (1-[3-dimethylaminopropyl]-3-

ethyl carbodiimide) for synthesizing a PLA-PEG-PLA multiblock copolymer from ABA polymers.

ii) Novel polyester-polyethylene multiblock copolymer and method for synthesizing the same

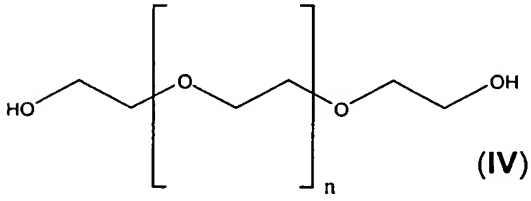
According to another aspect, the invention provides a multiblock copolymer that is composed of alternate blocks of polyester and of polyethylene glycol. According to the invention, these blocks are arranged according to the following manner:

ABA-B'- ABA-B'-ABA-B'-ABA (III)

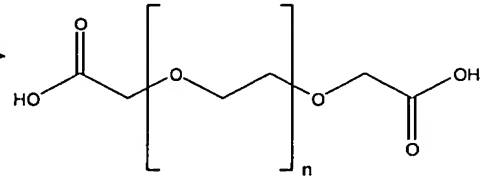
wherein A is a polyester, B is a polyethylene glycol and B' is a dicarboxylic polyethylene.

A non-exhaustive list of suitable polyesters includes polylactic acid (PLA); polylactic-co-glycolic acid (PLGA); polycaprolactone (PCL), and polyhydroxy butyrate. A non-exhaustive list of suitable polyethylenes includes polyethylene oxides (PEO) such as polyethylene glycol (PEG). A non-exhaustive list of suitable dicarboxylic polyethylene includes dichloride dicarboxylic PEG and dibromide dicarboxylic PEG. More preferably, the polyester consists of PLA, the polyethylene consists of PEG and the dicarboxylic polyethylene consists of dichlorine dicarboxylic PEG.

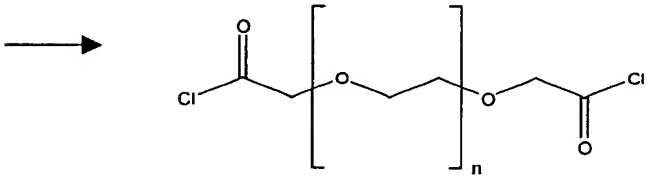
Preferably, the multiblock copolymer is synthesized by using PEG as the polyethylene. According to this embodiment, commercially available PEG is oxidized into a dicarboxylic PEG, then a dichloride acid is formed:



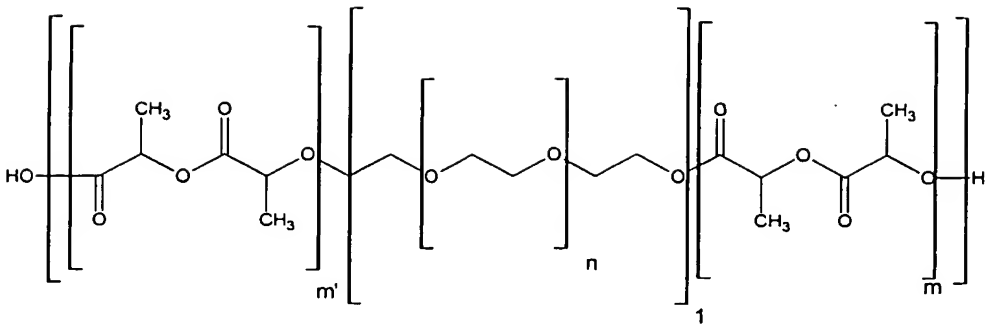
(IV)



(V)



(VI)



(VII)

According to this embodiment, these blocks are arranged according to the following manner:

ABA-c-ABA-c-ABA-c-ABA (I)

wherein "ABA" is the PLA-PEG-PLA triblock and "c" is a carboxylic diacid (e.g. butanedioic acid, propanedioic acid, pentanedioic acid (IUPAC nomenclature)).

According to another, more preferred embodiment, the multiblock copolymer is composed of alternate blocks of polyester and polyethylene glycol. According to this embodiment, these blocks are arranged according to the following manner:

ABA-B'- ABA-B'-ABA-B'-ABA (III)

wherein A is a polyester, B is a polyethylene glycol and B' is a dicarboxylic polyethylene.

A non-exhaustive list of suitable polyesters includes polylactic acid (PLA); polylactic-co-glycolic acid (PLGA); polycaprolactone (PCL), and polyhydroxy butyrate. A non-exhaustive list of suitable polyethylene includes polyethylene oxides (PEO) such as polyethylene glycol (PEG). A non-exhaustive list of suitable dicarboxylic polyethylene includes dichloride dicarboxylic PEG and dibromide dicarboxylic PEG. More preferably, the polyester consists of PLA, the polyethylene consists of PEG and the dicarboxylic polyethylene consist of dichloride dicarboxylic PEG.

ii) Polyester

Preferably, the nanospheres comprise about 0.1% to 99% of a polyester. The polyester, entangled with the multiblock copolymer, is useful for increasing the rigidity of the nanospheres. A non-exhaustive list of suitable polyester

described herein can be used in the practice for testing of the present invention, the preferred methods and materials are described.

Example 1: Synthesis and characterization of novel PLA-PEG multiblock copolymer

Introduction

Biodegradable polymers are studied in an increasing number of medical applications. They are used as drug carriers, controlled release systems, etc. Some authors are interested in the possibilities that a copolymer consisting of polylactic acid (PLA) and polyethylene glycol (PEG) can offer. A multiblock copolymer composed of PLA and PEG is of considerable interest as a drug carrier, since the PLA segments could provide rigidity, while the PEG portions confer stealth behavior (R.H. Muller. CRC Press Inc., Boca Raton, Florida, 1991: 45-46). PEG can offer a certain degree of hydrophilicity to the polymer that can be useful if we want to use it as a carrier for an hydrophilic drug. But the current ring-opening polycondensation of (D,L)-lactide in the presence of PEG can only produce an A-B-A triblock copolymer where the B block (PEG) is trapped between two A blocks (PLA).

We propose here an efficient synthesis method for a polyester-polyethylene multiblock copolymer where the polyester (A) blocks alternate with polyethylene (B) blocks to form a repetitive sequence.

Experimental methods

i) Materials

Polyethylene glycol (molecular weight 400), (D,L)-lactide, tetraphenyltin and adipic acid were purchased from Aldrich Chemical Company, Inc. (Oakville, Ont., Canada) and were dried under vacuum in the presence of phosphorus pentoxide for 24 hours prior to use. N,N-dimethylformamide was distilled over calcium hydride and kept on a 4Å molecular sieve prior to use. Thionyl chloride and pyridine were used as received from Aldrich Chemical Company.

ii) Preparation of triblock PLA-PEG-PLA copolymer

The triblock polymer was synthesized by a ring-opening polycondensation of (D,L)-lactide in the presence of PEG, as described by Cohn and Younes (*J. Biomed. Mater.Res.* **22**(11): 993-1009 (1988)). PEGs with different molecular weight were used. Briefly, 8.3 mmol of PEG (molecular weight 200, 400 or 1500) were added to 158.3 mmol of (D,L)-lactide (molecular weight 144,13) in a round bottom, single neck flask. Tetraphenyltin 0.01% was used as a catalyst. The reaction was carried at 180°C for 6 h under an argon-inert atmosphere. The resulting polymer was precipitated in water from acetone, removing any un-reacted PEG or (D,L)-lactide. The polymer was then dried under vacuum with phosphorus pentoxide.

iii) Preparation of a multiblock $(PLA\text{-}PEG\text{-}PLA)_n$ copolymer

The triblock copolymer (3 mmol) and adipic acid (3 mmol) were dissolved in N,N-dimethylformamide (40 ml) under an argon-inert atmosphere. A solution of thionyl chloride (15 mmol) in pyridine (15 ml) was added at 0°C over a period of 30 minutes. The temperature was brought to 20°C over a period of 10 hours, under magnetic stirring. The polymer was then precipitated in water and washed several times to remove any trace of solvent. Its structure is shown in Figure 1.

iv) Contact angle measurements

Contact angle measurements were made using a Tantec CAM-MICRO™ contact angle meter. For each copolymer, 200 mg was dissolved in 3 ml of dichloromethane, and a thin film was cast on a glass slide. The films were dried under vacuum to remove any trace of solvent. Polylactic acid was used as a reference for the contact angle measurement. Contact angle measurements were made at 0 and 420 seconds.

Results and discussion

^{1}H- NMR, using a Bruker 400 MHz spectrometer showed a typical spectrum for the triblock copolymer with peaks at 5.2 ppm corresponding to the tertiary PLA proton, at 3.6 ppm for the protons of the repeating units in the PEG chain, at 4.3

ppm for the PEG connecting unit to the PLA block, and at 1.5 ppm for the pendant methyl group of the PLA chain (not shown). For the multiblock copolymer showed in **Figure 6**, peaks corresponding to the protons in the adipic acid chain were detected at 3.0 and 2.3 ppm (see **Figure 7**).

Molecular weight (**Table 1**) was mesured by gel permeation chromatography using a Waters™ spectrometer. Molecular weight around 2000 Da for the triblock copolymer and 10 000 Da for the multiblock copolymer showed that the blocks were covalently bounded together.

Table 1: Molecular weight measurements

TRIBLOCK	Mn	Mw	I
PEG 200	1474.17	2151.45	1.46
PEG 400	900.84	1285.34	1.43
PEG 1450	2835.22	3595.42	1.27
MULTIBLOCK			
PEG 200	4357.02	9657.48	2.22
PEG 400	3646.71	8537.77	2.34
PEG 1450	6607.95	12040.6	1.82

Contact angle measurements (**Table 2**), show that the polycondensation of PLA with PEG reduce the contact angle thus augmenting the hydrophilicity of the copolymer compared to PLA alone.

Table 2: Contact angle measurements

Polymer	Contact angle (t = 0s)	Contact angle (t = 420s)
PLA	73.7	49.3
Multiblock (PEG 200)	59.6	38.6
Multiblock (PEG 400)	19.2	2.0
Multiblock (PEG 1450)	17.6	0.6

In computer simulation (**Figure 8**), the copolymer tends to show clear separation of the PLA and PEG domains. This spatial organization is confirmed by AFM Phase imaging microscopy of a copolymer film (**Figures 9** and **10**) showing a clear segregation between the PEG and PLA blocks.

Conclusion

PEG-ε-caprolactone form micelles easier than the triblock copolymers, hence the multiblock copolymer will possess enhanced efficiency for NS preparation. It is of growing interest to study the behavior of this new class of multiblock (-PLA-PEG-PLA-)$_n$ copolymer as a drug carrier for prolonged release of anti-infectious or anti-neoplasic drugs. Prior to be used as a new biomaterial, cytocompatibility and degradation studies must be conducted for safety.

Hence, the objectives of this study were to 1) conduct *in vitro* cytotoxicity tests on the new biomaterial; 2) manufacture NS from the (-PLA-PEG-PLA-)$_n$ multiblock copolymer; and 3) report the physico-chemical properties of the NS with regard to the size, zeta potential, porosity and hydrophilicity. Furthermore, incorporation of Rhodamine B as a drug model in the NS and its *in vitro* release were studied to assess the potential of these NS as a drug carrier.

Materials and Methods

Materials

Rhodamine B was purchased from Sigma (St Louis, MO, USA). Chloroform was obtained from Anachemia (Montreal, Qc, Canada). Poly (vinylalcohol) 80% hydrolyzed, sodium hydrogenophosphate 98%, sodium chloride 98%, and sodium azide were from Aldrich Chemical Company Inc., Minimum Essential Medium, Pyruvate substrate, Sigma color reagent, gentamycin, and MTT (dimethyl thiazoldiphenyltetrazoliumbromide) were from Sigma (St Louis, Mo, USA). Hanks' Balanced Salt Solution, fetal bovine serum, and trypsin-EDTA were obtained from Gibco Life Technologies (Burlington, Canada). Tetraphenyltin, adipic chloride, and pyridine were purchased from Aldrich (Oakville, ON, Canada).

2) Polymer synthesis

A triblock polymer was first synthesized by a ring-opening polycondensation of (DL)-Lactide in the presence of polyethylene glycol (PEG), as described by Cohn and Younes (*J. of Biomredical Materials Res.* **22**: 993-1009 (1988)). Briefly, 8.3 mmol of PEG (molecular weight 400) was added to 158.3 mmol of (DL)-Lactide (molecular weight 10000) in a round bottom single neck flask.

10. The stealthy polymeric biodegradable nanospheres according to claim 7 wherein said polyethylene is a polyethylene oxide (PEO).

11. The stealthy polymeric biodegradable nanospheres according to claim 10, wherein the polyethylene oxide (PEO) is a polyethylene glycol (PEG).

12. The stealthy polymeric biodegradable nanospheres according to any one of claims 7 to 11, wherein the dicarboxylic polyethylene is selected from the group of dichloride dicarboxylic (PEG) and dibromide dicarboxylic PEG.

13. The stealthy polymeric biodegradable nanospheres according to any one of claims 1 to 12, wherein the polyester (ii) is selected from the group consisting of polylactic acid (PLA), polylactic-co-glycolic (PLGA), polycaprolactone (PCL) and their copolymers.

14. The stealthy polymeric biodegradable nanospheres according to claim 13, wherein the polyester (ii) is polylactic acid (PLA).

15. The stealthy polymeric biodegradable nanospheres according to any one of claims 1 to 14, wherein the pharmaceutical compound (iii) is a drug, a protein and/or a nucleic acid molecule for the prevention or treatment of various diseases and/or delivery of different types of therapeutic agents.

16. The stealthy polymeric biodegradable nanospheres according to claim 15, wherein the therapeutic agents are selected from the group consisting of anticancer agents, immunosuppressive agents, agents for steroid therapy, anti-arrhythmic agents, antibiotics, antiparasitics, antivirals, antifungics, gene-therapy agents, antisense molecules, orphan drugs, and vitamins.

17. The stealthy polymeric biodegradable nanospheres according to any one of claims 1 to 16, wherein the nanosphere has an average size of less than 800 nm.

26. The polyester-polyethylene multiblock copolymer according to claim 25, wherein the polyethylene oxide (PEO) is a polyethylene glycol (PEG).

27. The polyester-polyethylene multiblock copolymer according to any one of claims 22 to 26, wherein the dicarboxylic polyethylene is selected from the group consisting of dichloride dicarboxylic (PEG) and dibromide dicarboxylic PEG.

28. A method for preparing the polyester-polyethylene multiblock polymer of formula (III) as defined in any one of claims 22 to 27, comprising the steps of:

a) oxidizing both terminal hydroxyl groups (-OH) of a polyethylene glycol into corresponding carboxylic groups (COOH) by means of a Jones reaction;

b) chlorinating the carboxylic functions of the polyethylene glycol obtained in step a) by making use of a $SOCl_2$ reagent so as to obtain a polyethylene glycol with terminal dichloride acid functions; and

c) reacting the polyethylene glycol having terminal dichloride acid functions obtained in step b) with the PLA-PEG-PLA triblock polymer obtained in claim 34 by making use of polycondensation reaction so as to obtain a multiblock copolymer as claimed in any one of claims 3 to 12.

29. An improved method for preparing a PLA-PEG-PLA multiblock copolymer of formula (I):

$$ABA\text{-}(c\text{-}ABA)_n\text{-}c\text{-}ABA \qquad (I)$$

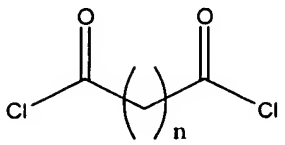
wherein

- n is a number equal or higher than 2;
- ABA is a PLA-PEG-PLA triblock; and
- c is a carboxylic diacid.

said method comprising the steps of:

a) preparing a PLA-PEG-PLA triblock;

b) mixing the PLA-PEG-PLA triblock prepared in step a) with a diacid of formula (II):



(II)

wherein n is a number equal to or greater than 1; and

c) subjecting the mixture of step b) to a polycondensation reaction with the presence of a dicyclohexylcarboxydiimide reagent and/or a chemical equivalent thereof, said catalyst improving the efficiency of the reaction, thereby allowing to obtain the requested multiblock copolymer.

30. The method according to claim 29, wherein step a) comprises the steps of:

(i) reacting at least one monomer A with at least one monomer B by a polycondensation reaction so as to produce a PLA-PEG-PLA triblock;

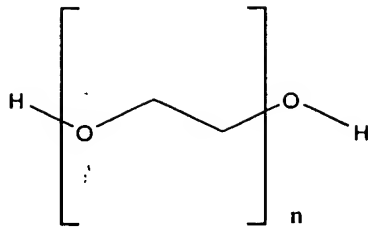
(ii) dissolving the PLA-PEG-PLA triblock obtained in step (i) in acetone;

(iii) precipitating the dissolved PLA-PEG-PLA triblock in step (ii) in water; and

(iv) washing and drying the PLA-PEG-PLA triblock polymer.

31. The method according to claim 30, wherein monomer A is selected from the group comprising of dioxanediones, lactones and dioxanones.

32. The method according to claim 30 or 31, wherein monomer B is a polyethylene glycol (PEG) represented by the formula (B):



wherein n represents a number between 200 and 2000.

33. The method according to any one of claims 30 to 32, wherein step (ii) is carried out with a tin based catalyst at a temperature between 160° C and 180° C under an inert atmosphere.

34. The method according to any one of claims 29 to 33, wherein the diacid chloride used in step b) is selected from the group comprising of propanedioic acid, butanedioic acid and pentanedioic acid.

35. The method according to any one of claims 29 to 34, wherein the chemical equivalent of dicyclohexylcarboxydiimide (DCC) is 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC).

36. The method according to any one of claims 29 to 35, wherein the carboxylic diacid in step c) is selected, the group comprising of butanedioic acid, propanedioic acid and pentanedioic acid.

37. A method for delivering a pharmaceutical compound into a mammal, said method comprising the step of:

administering to the mammal a stealthy polymeric biodegradable nanosphere as claimed in any one of claims 1 to 20 loaded with a therapeutically effective amount of the pharmaceutical compound.

38. The method according to claim 37, wherein the pharmaceutical compound comprises a therapeutic agent which is selected from the group of anticancer agents, immunosuppressive agents, agents for steroid therapy, anti-arrhythmic agents, antibiotics, antiparasitics, antivirals, antifungics, gene-therapy agents, antisense molecules, orphan drugs, and vitamins.